CATALYTIC BUFFERING: DEVELOPMENT OF THE FLUORIDE-RESISTANT UREASES OF *Klebsiella pneumoniae*

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ABSTRACT

Catalytic buffering is an advanced method of pH control for the enzyme-based chemical agent decontamination technology Advanced Catalytic Enzyme System (ACES). Ammonia production from urea by urease neutralizes the production of *O*-alkylphosphonic acids resulting from OPAA and OPH catalytic hydrolysis of G-agents and VX. Unfortunately, ureases are inhibited by low levels of fluoride, another product of GB, GD and GF hydrolysis. To overcome this problem, the urease structural genes of the enteric bacterium *Klebsiella pneumoniae* were isolated from their accessory genes and randomly mutagenized to produce ureases with superior fluoride-resistance (FR). Mutagenized *ureABC* genes were co-transformed with another plasmid containing the accessory genes (*ureDEFG*) needed for nickel incorporation into *E. coli* to produce active urease in the clones. *E. coli in vitro* mutagenesis was followed by screening of the lysed, double-plasmid clones for fluoride-resistant urease activity. Several FR clones were isolated from this procedure with greatly enhanced activity in 0.1 M fluoride after successive generations of mutagenesis. Successive generations of urease mutants showed higher levels of fluoride resistance. These results indicate that *in vitro* mutagenesis can be used to successfully generate fluoride-resistant ureases needed for the catalytic buffering method of pH control.

INTRODUCTION

Catalytic buffering is an advanced pH control method. It relies on a catalyst, e.g. enzyme, to produce ions from a substrate to regulate the pH of the solution in question. Catalytic buffering is particularly attractive to the Advanced Catalytic Enzyme System (ACES) because the acidic *O*-alkylphosphonate (EMPA, IMPA, etc.) products of the OPAA- and OPH-mediated G and V agent hydrolysis dramatically lowers the reaction pH. Catalytic buffering would create an on-demand neutralization system without the

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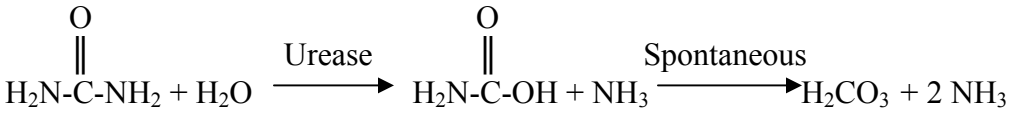
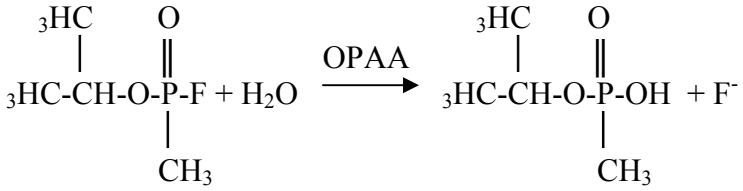
use of additional harsh chemicals to maintain the desired reaction pH for agent detoxification enzymes.

An example of a possible catalytic buffering system is the ammonia production caused by the hydrolysis of urea by Urea Amidohydrolase (Urease):



Urease is nickel metalloenzyme widely distributed in nature (Mobley, et al., 1995). Urea is a neutral molecule that does not affect the pH of the aqueous solution. Ammonia production by urease raises the solution pH. This rise in pH produced by urease activity counters the pH decrease arising from the O-alkylphosphonate products of OPAA or OPH catalysis of organophosphorus nerve agents. While this seems an appropriate enzyme for catalytic buffering, urease is inhibited by low levels of fluoride ion (Todd and Hausinger, 2000). Fluoride ion is one of the products of GB hydrolysis:



The sensitivity of urease to fluoride ion is widespread and occurs in ureases isolated from any source (bacterial, animal, plant). The amino acid sequence of these enzymes is fairly well conserved, so all of these enzymes share similar biochemical properties.

To address the sensitivity of urease to fluoride ion, efforts were initiated to find a naturally-occurring urease or produce one through enrichment culture of environmental samples. These experiments made use of urea as a sole nutritional nitrogen source available to the microbes along with fluoride in the culture medium. Many attempts were made to produce a fluoride resistant (FR) urease through microbial enrichment, but these efforts were not successful. Bacteria were isolated that would grow on urea as a sole nitrogen source in the presence of fluoride ion, but the urease activity found the intracellular extracts of these cells was still fluoride-inhibited.

Since the enrichment culture results indicated that is was unlikely that a FR urease would be found in nature, mutagenesis of a cloned urease to increase its fluoride resistance was selected as an alternative solution. The urease of the gram-negative enteric bacteria *Klebsiella pneumoniae* (formerly *aerogenes*) was chosen for these studies. The KAU urease is cloned, well characterized and it's fluoride sensitivity mechanism determined (Todd and Hausinger, 1987; Mulrooney and Hausinger, 1990; Lee et al., 1992; Todd and Hausinger, 2000). The structure of the KAU operon consists of three structural genes (*ureABC*) and four accessory genes involved in nickel uptake and incorporation (*ureDEFG*).

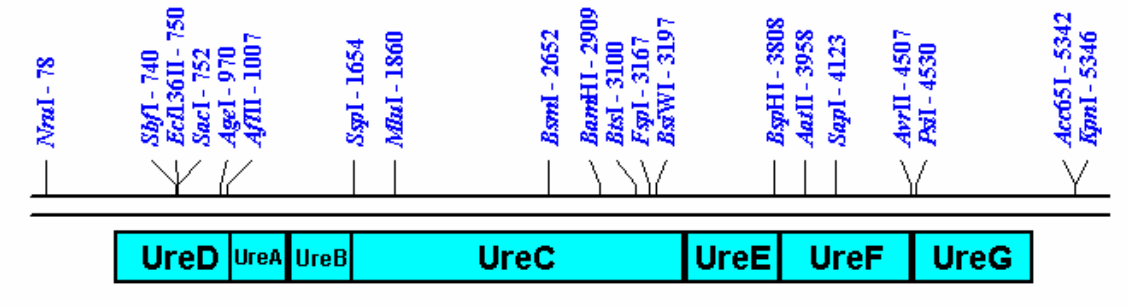


Figure 1. The KAU gene cluster. Restriction enzyme locations are noted in base pair distances from the cluster origin.

Biophysical evidence of the mechanism of fluoride sensitivity (Todd and Hausinger, 2000) did not contain sequence specific details that could be used for site-directed mutagenesis, so random mutagenesis of the structural genes (*ureABC*) was pursued. The method chosen to introduce these mutations, *in vitro* mutagenesis, creates random DNA point mutations using an *E. coli* mutator strain. The FR mutant plasmids isolated from this treatment can then be transformed again into this strain, fluoride-resistant (FR) clones isolated and the process repeated to create multi-generational mutants with greatly enhanced activity. This report outlines the development of FR KAU ureases using *in vitro* mutagenesis and their kinetic analysis.

MATERIALS AND METHODS

The plasmids pKAU17 and pKAUDEG were donated by Dr. Scott Mulrooney of the University of Michigan, Lansing. *E. coli* strains used in these experiments were DH10B (Invitrogen) and XL1-red (Promega). DH10B was used as the screening and cloning host for the plasmids; XL1-red was used for mutagenesis only. The cloning vector used for these experiments was pCM66, a dual replicon, broad host range, mobilizable, kanamycin-resistant plasmid (Marx and Lindstrom, 2001). Growth media were Luria agar (LA), Luria agar/broth + 0.4 mM $NiCl_2$ (LA_{nick} or LB_{nick}) or M9 Modified Urea agar or broth (M9 Mod Urea). Antibiotic selection for the plasmids was 50 µg/ml ampicillin (pKAUDEFG or pKAU17) and/or 25 µg/ml kanamycin (pCM66-derived plasmids). Bluogal was added to the LA to provide blue/white color screening needed for recombinant plasmid identification. Kinetic assay medium was 0-0.2 M Fluoride in 0.5-1.25 mM HEPES, pH 7, 55 µg/ml Phenol Red; urea was added at a final concentration of 10 mM to initiate the reaction. Screening solution was 0.1-0.2 M sodium fluoride in 0.5-1.25 mM HEPES, pH 7, 55 µg/ml Phenol Red, 10 mM HEPES. Pre-inhibition solution for the screening was 0.5-1.25 mM HEPES, pH 7, 0.1 M sodium fluoride. Screening filters were 82 mm Nitrocellulose circles. Screening pads were VWR 283 (Ahlstrom) cut to size. Rubbermaid supplied the large polycarbonate screening boxes (18 X 26 X 3 ½") for the mass initial screening.

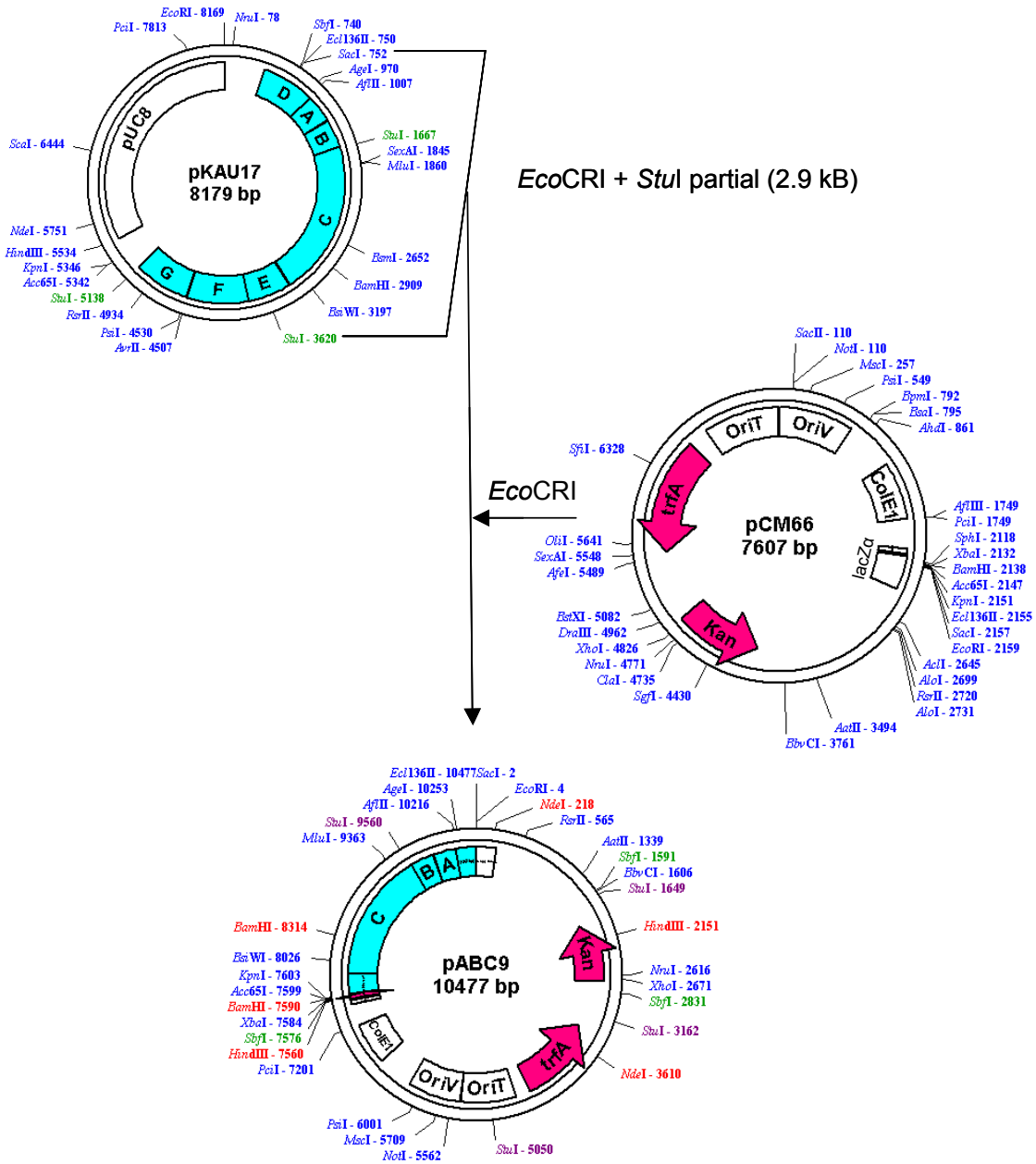


Figure 2. Cloning of pABC9 from pKAU17 and pCM66. KAU urease genes are shown as DABC, etc. in the turquoise boxes.

Subcloning of *ureABC* into pCM66

The *ureABC* genes of pKAU17 were removed with a sequential *Eco*CRI (neoschizomer of *Sac*I) and *Stu*I (partial) double digest and cloned into the *Eco*CRI site of pCM66. A single orientation of the *ureABC* genes counter to the lac promoter of pCM66 was obtained (pABC9) from the blunt end ligation (Figure 2). The integrity of this construct was confirmed by co-transformation of pABC9 with pKAUDEFG into *E. coli* DH10B to produce active urease in the double plasmid clones and testing in M9 Mod

Urea agar and broth. Formation of bright pink color in the media (OD590) was indicative of Ure^+ clones. Inclusion of fluoride in the medium (0.1-0.2 M) inhibited growth and color formation, but eventually both would occur over extended incubation.

Mutagenesis of pABC9

The pABC9 plasmid was transformed into competent XL1-red cells (Promega). The cells were spread onto LA Kan_{25} and incubated for 48 h at 37°. The colonies were scraped from the plates and the plasmids extracted using the Wizard SV procedure (Promega).

Screening of Mutagenized pABC9

Mass Initial Screening

The mutagenized plasmids were co-transformed with pKAUDEFG into Electromax DH10B, diluted and spread onto 40, numbered LA_{nick} Kan_{25} Amp_{50} plates. After overnight incubation at 37°C, the colonies were lifted from the plates with numbered nitrocellulose filters. The filters were placed colony side up into the bottom of water-dampened glass Petri dishes. The lifted plates were returned to 37° to re-grow the colonies. The colonies on the filters were lysed with 2 ml of chloroform in the lids of the inverted glass Petri dishes for 30'. The lids were removed from the plates and the residual chloroform evaporated for 2' in a fume hood. The filters were laid on pads soaked with pre-inhibition solution for 10', RT in a large screening box. After pre-inhibition, the filters were transferred to pads soaked with the assay solution in another large screening box. The pink color development was noted at RT from 30" to 10'. Lysed, Ure^+ positive colonies were tracked via number and location on the filters to the corresponding plates.

Patch Screening

After the colonies re-grew on the lifted plates from above, the positive colonies were picked and patched onto LA_{nick} Kan_{25} Amp_{50} plates. The patched plates were incubated overnight at 37°. The original patch plate was replica plated with velveteen onto three LA_{nick} Kan_{25} Amp_{50} plates. After overnight growth, the replicated patches were lifted from plates with filters, lysed and assayed in the same manner described above but assayed in individual polystyrene plates instead of the large box. The assay solution used for this analysis varied in the fluoride (0.1-0.2) and in the buffer (0.5-1.25 mM HEPES) concentration to detect the best FR activity of the patched clones. Clones with the best activity in the highest buffer and fluoride concentration were picked from the original patch plate, inoculated into 5 ml of LB_{nick} Kan_{25} Amp_{50} and the cultures incubated overnight at 37°C. This culture served as the source for the inoculum for the crude extract preparation, the frozen stock and the mini plasmid prep.

Intracellular Extract Preparation

1 ml of the 5 ml FR broth culture from above was used to inoculate 100 ml of LB_{nick} Kan_{25} $Amp_{50.}$ This culture was incubated for 24 h, 220 rpm, 37°C. The cells were

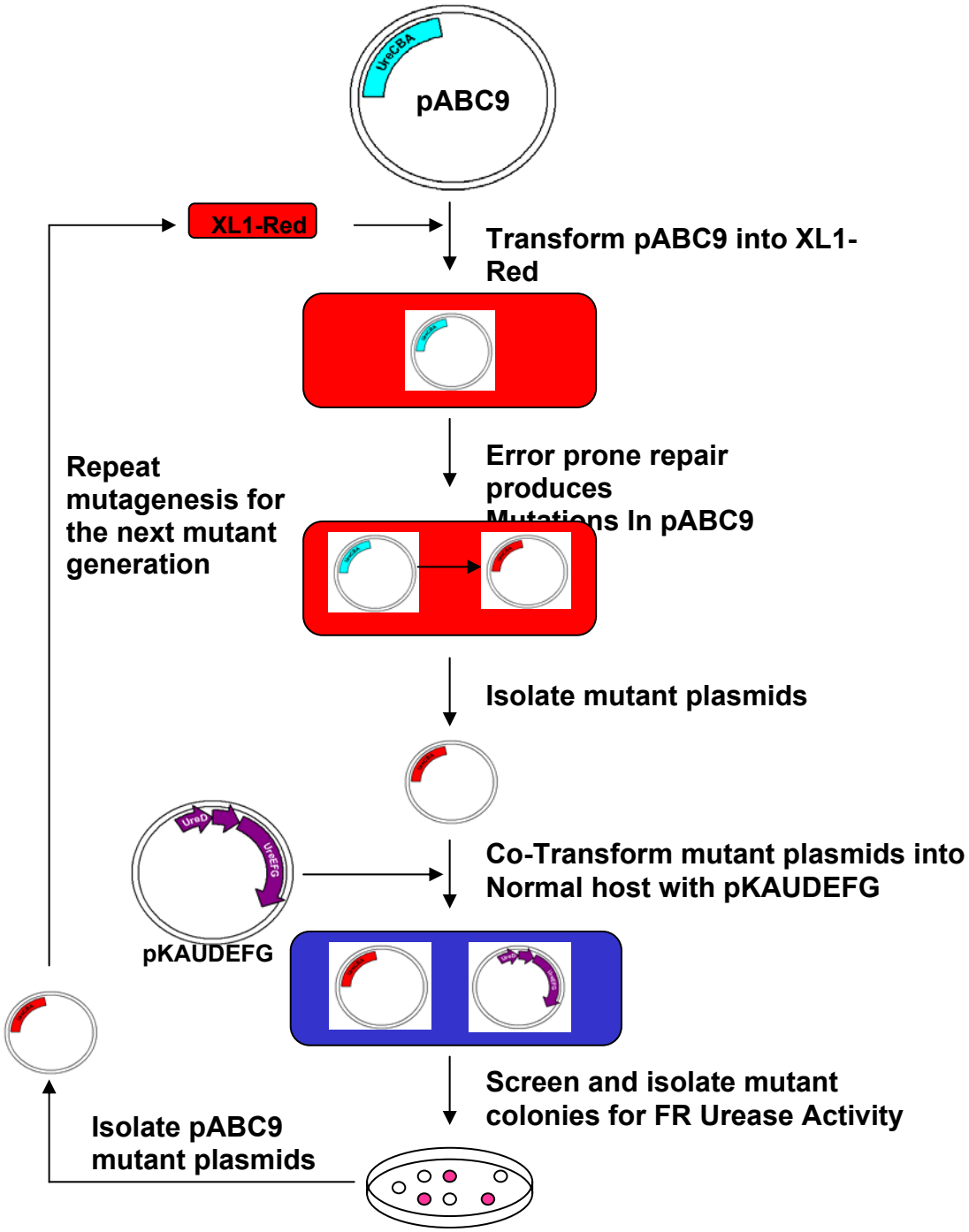


Figure 3. *In vitro* mutagenesis of the KAU structural gene cluster

harvested, washed once in 0.5 mM HEPES, pH 7, 4°, and resuspended in 0.5 ml of the same buffer. The cells were broken in a mini-bead beater and the intracellular extract clarified at 20,000 X g at 4°, 5'. The clarification process was repeated once to remove all traces of cells and glass beads.

Intracellular Extract Analysis

Protein content of the intracellular extract was determined with the Coomassie Blue Plus reagent (Pierce). The intracellular extracts were screened for FR urease activity in microplate format with the same assay solution used for screening and pre-incubated with the same solution minus the urea substrate. For these pre-incubation reactions the substrate, 0.2 M urea, was added to the wells to a final concentration of 10 mM to initiate the reaction. The reaction progress was captured digitally at 5' and 10' intervals. Wells were also included in the assay that contained the crude extracts of the wild type KAU urease (pABC9) and/or the parent source crude extract for that generation of mutant. Those crude extracts showing the best FR activity in microplate format were assayed in 1 ml final reaction volume using a Perkin-Elmer Lambda 25 spectrophotometer at 590 nm with an 8-cell changer and the KinLab program. The pre-incubation solutions for the kinetic analysis contained 0, 0.025, 0.05 and 0.1 M F. Reaction rates were recorded as A_{590}/min. Reduced kinetic data was calculated in MS Excel as A_{590}/min-mg protein. Activity comparisons were made by dividing the mutant activity with that of the parent and/or the wild type, resulting in data presented in multiples of the progenitor activity.

Capture of Single Plasmid Clones from Double Plasmid Clones

The pABC9 mutant plasmids were recovered from the double plasmid clones resulting from the FR screening by clipped transformation. This was necessary because pABC9 mutant genes are altered separately in succeeding generations in XL1-Red from the accessory proteins, which reside on another plasmid, pKAUDEFG. Linearized plasmids do not result in transformed clones in most cases, so the pKAUDEFG plasmid is separated from pABC9 in this manner. The FR mini plasmid preps prepared after the patch screening were digested with the restriction enzyme, *Sca*I, which cuts once in pKAUDEFG but not in pABC9. The digests were transformed into competent DH10B cells and the transformed cells plated on LA Kan_{25}. Colonies obtained from this transformation were tested for the absence of the pKAUDEFG plasmid by picking and patching them onto LA Amp_{50} plates and inoculating them into M9 mod Urea Kan_{25}Broth. Colonies with the $Kan^{r}Amp^{s}Ure^{-}$ phenotype were inoculated into 5 ml of LA Kan_{25}, shaken overnight at 37°, harvested, and plasmids prepared from the cells using the Wizard SV procedure. Digestion with BamH1, *Eco*R1-*Hind*III and subsequent agarose gel electrophoresis were used to confirm the presence of the sole pABC9 descendent plasmids.

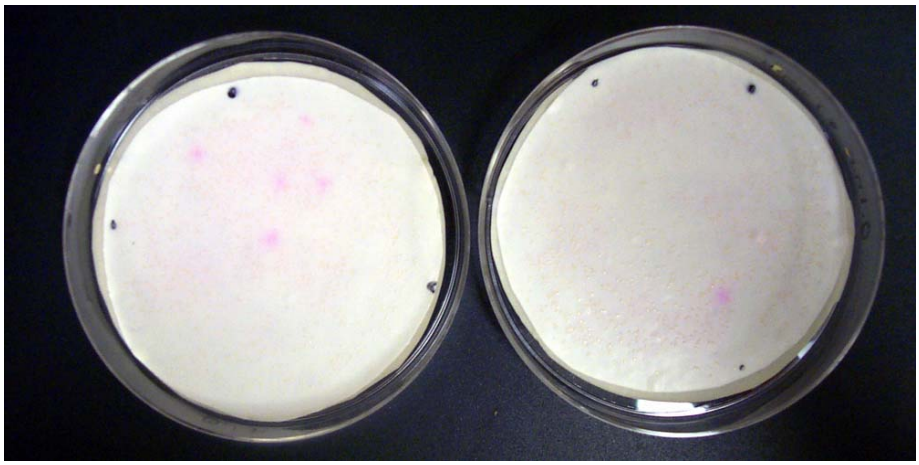


Figure 4. Lysed cell filter screening assay. Intense pink areas on the filter are the result of FR urease activity (increased pH) from lysed colonies in the presence of 0.1 M F.

RESULTS

Subcloning of *ureABC*

Transfer of the *ureABC* genes from the complete Urease gene cluster of pKAU17 was necessary to separate the urease structural genes from the accessory genes. It was theorized that getting simultaneous productive or neutral mutations in both sets of genes (7 total) was not statistically probable. Previous work by Hausinger's Lab with KAU indicated that active urease could be produced from having the genes on separate plasmids. In addition, active urease production using structural genes from one species and accessory genes from another was reported previously, presumably due to the high degree of Urease sequence conservation. *UreABC* was successfully subcloned into the broad host range vector pCM66 (Marx and Lindstrom, 2000) using the scheme outlined in Figure 2. This plasmid also contained two replicons (IncP and ColE1), a feature we believed useful because of the risk of losing one replicative function due to the random nature of the *in vitro* mutagenesis, which creates mutations in the vector, its insert and the XL1-Red chromosome. The double plasmid clones of pABC9 and pKAUDEFG produced active urease in *E. coli*. The pABC9 urease genes use their own promoter, not the P_{lac} promoter of pCM66.

Screening Double Plasmid Clones for FR Urease Activity

Photographs of FR urease lysed colony filter assay are shown in figure 4. The background appears light yellow with the lysed FR colonies an intense pink. This reaction is easily detected by eye, so locating the correct colony is straightforward. Patching the resulting re-grown colonies from the lifted plates allows replication of the

patches to other plates, whose lysed colonies can in turn be assayed under different stringency conditions to find the best FR mutants.

Nutritional selection of the FR clones with urea as a sole nitrogen source and fluoride in the growth medium was initially envisioned as the screening procedure. This was not possible, because the bacteria readily mutate their ability to take up fluoride, and will grow well and produce ammonia from urea simply because the fluoride cannot enter the mutant cells. Because of this natural phenomenon, the vast majority of cells recovered after growth in M9mod Urea medium with fluoride do not contain fluoride-resistant ureases.

This dilemma was solved by using the lysed colony filter assay method to accomplish several screening requirements: 1) All the cells must lyse 2) fluoride must be able to freely contact the urease, an intracellular enzyme 3) Pre-inhibition by fluoride without substrate and pH indicator 4) more than one type of assay solution can be applied successively after pre-inhibition 5) reaction products must be visible to the eye to detect a FR urease and 6) viable cells must be available to provide an inoculum after the screening for subsequent plasmid isolation and microbial culturing and 7) several thousand colonies can be screened at once. Several lysis methods were tried, but chloroform lysis on nitrocellulose filters worked the best. Pre-inhibition by fluoride ion without urea was found necessary for the screening because fluoride is a slow acting inhibitor; some level of activity will occur in all the mutant clones with fluoride, and this burst of initial activity results in a high background.

Successive generations of mutations required more rigorous conditions to separate the high activity FR mutants from the several thousand other lysed colonies on the filters. Although just a few colonies would be FR in the first generation, in the second generation virtually all of them are positive if assayed with the same stringency as the first generation. Increasing the buffer concentration in the pre-incubation and assay solutions was the most successful method found to raise the stringency of the assay. This works well because FR ureases are detected by a rise in the assay solution pH surrounding the lysed colony. The higher the buffer concentration, the more ammonia must be produced to raise the pH and change the phenol red indicator from orange to pink.

Kinetic analysis

Kinetic urease activity analyses of the FR mutant crude extracts confirmed the elevated activity of these enzymes when the fluoride concentration was 0.025 to 0.1 M. This activity varied among the mutants (Table 1), but was always higher than the wild type activity at all fluoride concentrations. Uninhibited urease activity was also usually much higher in the mutants than in the wild type. Mutants 13 and 901 showed the best FR urease activity of the 1st mutant generation, so the urease plasmids were isolated from these two clones and subjected to another round of *in vitro* mutagenesis. Second generation 13m or 901m mutants subsequently gave higher activity than their parent mutants, as is shown in Figure 5. Mutants 21-1(13m) and 38-3 (901m) were clearly the

best mutants found in the second generation. Mutant 9-1 was the best 3rd generation mutant obtained from 21-1.

Table 1. Crude Extract Urease Activity of *in vitro* Mutants in 0-0.1 M F. Activities are expressed as A_{590}/min-mg protein.

Mutant Extract	0 M F	0.025 M F	0.05 M F	0.1 M F
33-3	34.31	0.38	0.26	0.03
34-1	7.13	0.04	0.016	0.007
W+	0.65	0.003	0.001	0.001
7--1	11.99	0.23	0.17	0.03
21-1	17.80	0.24	0.17	0.12
38-3	10.07	0.29	0.11	0.05
FR13	3.37	0.05	0.02	0.002
FR901	0.37	0.001	0.001	0.001
33-3	34.31	0.38	0.26	0.03
34-1	7.13	0.04	0.016	0.007
7-1	11.99	0.23	0.17	0.03
9-1	34.86	3.73	1.27	0.63

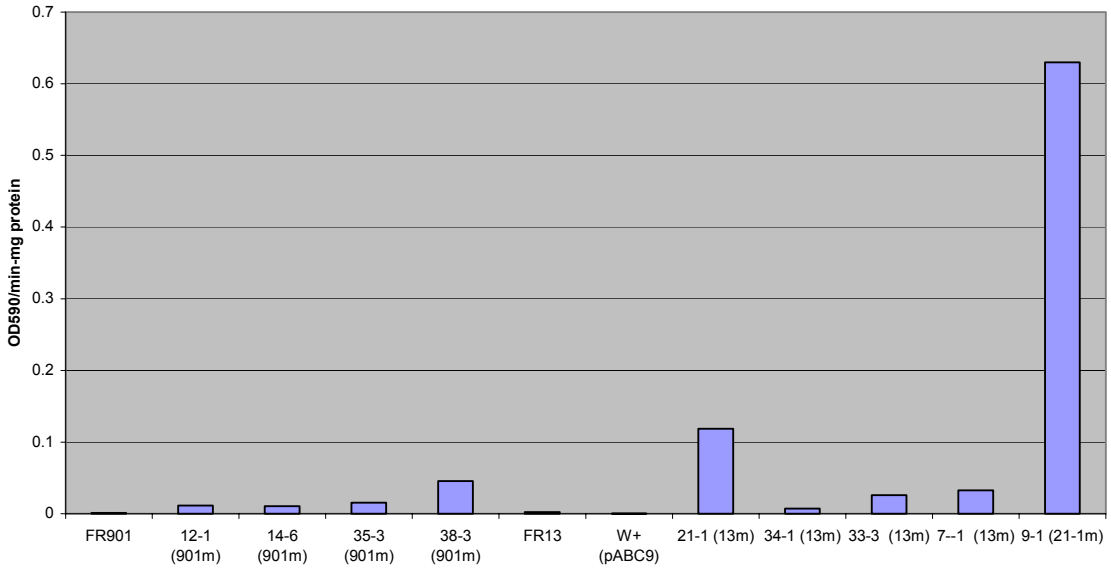


Figure 5. Comparison of wild type, 1st and 2nd generation mutant crude extract urease activities in 0.1 M F. The wild type is the unmutated pABC9 clone. FR 13 and FR 901 are 1st generation mutants. 21-1, 34-1, 33-3 and 7-1 are 2nd generation mutants of FR13. 12-1, 14-6, 35-3 and 38-3 are 2nd generation mutants (parent FR901). 9-1 is a 3rd generation mutant (21-1 parent).

CONCLUSIONS

In vitro mutagenesis combined with lysed colony filter screening of the KAU *ureABC* structural genes was used successfully to produce and identify fluoride-resistant (FR) mutant ureases. A simple random mutagenesis procedure was used in multiple generations to produce ureases with increased fluoride resistance. The best FR mutant isolated so far, 9-1 (21-1m), shows an increase of urease activity in 0.1 M F 133X times that of the wild type.

Information garnered from this study provided insight on previous attempts to produce fluoride resistant ureases by enrichment culture. It was learned that nutritional screening could not be used to screen for FR ureases in *E. coli* because they would apparently form fluoride permease mutants that block the influx of fluoride into the cells. In addition, the highly diffusible ammonia produced by the permease mutants feeds the surrounding non-permease mutants. Because of the ammonia release, little selectivity is offered by this screening technique, especially in liquid culture. Several urease-producing bacterial species from our microbial collection were screened initially and showed the same results. Eventually spontaneous mutation would allow them to grow in fluoride medium, but their ureases were not fluoride-resistant. This could readily explain why previous studies were unable to find FR ureases by enrichment culture, since extended periods of culturing in the same medium required in this procedure would allow ample time for these spontaneous permease mutations to occur. Using the lysed colony filter assay allowed the inhibitor to readily access the target enzyme without the outer cell structure barrier.

Urease mutagenesis is ongoing to produce enzymes with even higher levels of fluoride resistance than the current mutants for Catalytic Buffering applications. Future mutagenesis studies are planned that include enhancing urease performance in oxidizing reagents such as hydrogen peroxide.

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